

The University of Chicago

PURE CULTURES OF RABBIT
THYMUS EPITHELIUM

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE
DIVISION OF BIOLOGICAL SCIENCES IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY

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FIFTEEN FIGURES

INTRODUCTION

This report is a description of the behavior of rabbit thymus epithelium in pure culture. Such epithelium did not produce macrophages, although activity somewhat suggestive of phagocytosis was observed. On 2 occasions a cell somewhat resembling a lymphocyte was formed, but in general the free cells did not resemble connective tissue of any kind. In the mixed thymus cultures from which pure epithelium was isolated, the thymocytes exhibited most of the properties of true lymphocytes. The most informative observations were made with the aid of a time lapse moving picture apparatus.

Since the earliest descriptions of the origin of the thymus from the third entodermal pouch, there has been continued controversy over the nature of this unusual organ. The dispute concerns chiefly the nature and origin of the small cells, the disposition of the original entodermal epithelium, and the relation of the small cells to the stroma cells.

One source of this wide variance of opinion lies in the morphological similarity between the thymus stroma cells and

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those of the connective tissue. This similarity is not enough to establish the connective tissue origin of the cellular reticulum of the thymus. The problem might be clarified by demonstrating some of the actual and potential functions of these cells in tissue culture. But such a function as the ability to form lymphocytes, cannot be proved in mixed cultures where the origins of particular cell types are uncertain. This uncertainty is, in my opinion, inherent in the work of previous investigators with mixed cultures of the thymus (see discussion). The relationships between the various cells might become apparent in a pure culture of thymic stroma. Such pure cultures were obtained and will be described below.

MATERIAL AND METHODS

Small pieces of thymus from rabbits varying in age from 1 day to a year were cultured in heparinized rabbit plasma, and extract from 15- to 18-day rabbit embryos. The extract was the supernatant liquid from a centrifuged mixture, in approximately 4:1 proportions, of Tyrode solution and a mash prepared by forcing eviscerated and beheaded embryos through a Rosenow crusher. The cultures were planted in 1 drop each of plasma and extract and incubated at 37°C. on double cover slips according to the method of Maximow. Seventeen series were run with about 40-50 cultures in each series, making a total of about 700 cultures. They were maintained for periods varying from 1 to 6 weeks. Pure culture of thymus epithelium was obtained in about 60 different cultures in 11 of the 17 series.

The thymus was removed aseptically through the anterior chest wall avoiding the pleural mesothelium. To remove any of the latter which may have been included, all surfaces of the extracted bit of thymus were cut away and only the central portion was used for the cultures.

After 1 or more subcultures of small pieces rich in epithelium, and from which most of the free cells were gone, it was possible to cut away tongues (fig. 1) or portions of sheets of pure epithelium. If these were lifted and planted on

a fresh clot, a relatively short period of growth by extension into the clot was invariably followed by liquefaction and rounding up of the culture (fig. 8) with no further growth. Previous attempts in this laboratory to maintain growing cultures of pure thymus epithelium had failed for this reason. If, however, the bit of epithelium were left in place, the rest of the culture removed, and fresh plasma added, growth was sustained for long periods.

The living cultures were observed under the microscope at magnifications up to $900\times$ by use of thin cover glasses and an oil immersion objective of long working distance. The most promising were photographed with the aid of a time lapse photomicrographic apparatus using a 4-mm or 8-mm objective and with from 2 to 60 exposures per minute. The films were projected and studied at normal projection speed which resulted in apparent activity of 32 to $480\times$ the normal speed. During the photographing the cultures were maintained at about 37°C . by an incubator surrounding the microscope.

The following tissues were grown under similar conditions and photographed for comparison with the thymus cultures: subcutaneous connective tissue from adult and newborn rabbits; lymph nodes from rabbits 1 day to 2 weeks old; embryonic rabbit mesenchyme; gall bladder and intestinal epithelia from newborn rabbits.

The fixed and stained material consisted of sections and whole mounts of mixed and pure cultures, fixed in Zenker-formol and stained with hematoxylin-eosin-azure II. The cultures for sectioning were first embedded in thin celloidin and then cut away from the cover slip with a new razor blade. The resulting piece of celloidin and culture was re-embedded in celloidin and sectioned at 8μ . A few cultures were fixed in Regaud and stained with iron hematoxylin for mitochondria, and several were fixed by a modified Kopsch method for Golgi apparatus. Others were stained in vitro with neutral red and with Janus green and a few were impregnated with silver for reticular fibers.

Control pieces of thymus from most of the animals used for the cultures were fixed in Zenker-formol, sectioned at 8μ and stained with hematoxylin-eosin-azure II.

OBSERVATIONS

Living cultures

In all cultures the most striking activity in the first 24 hours is the migration of free cells into the fibrin where they undergo certain hypertrophic and retrogressive changes. At about 24 hours after planting, however, connective tissue spindle cells and epithelial sheets begin to grow out. The connective tissue cells form an irregular branching network while the epithelium grows in broad sheets or tongues (fig. 12). Although these cell types are usually intimately associated, under certain favorable conditions portions of tongues or sheets of pure epithelium were isolated and maintained.

The details of the changes observed in living cultures will be described under 4 heads: (A) Migrating cells in thymus culture, (B) epithelium in thymus culture, (C) pure cultures of epithelium of thymus, gall bladder and intestine, and (D) connective tissue cultures.

The description is confined principally to those cells in the zone of outgrowth since the explanted mother piece is too thick to permit detailed observation or photography.

Migration of free cells. The great mass of the free cells in the zone of migration in the earliest cultures are thymocytes. The term thymocyte is used advisedly to avoid raising, for the time being, the question of their origin and nature. Typical actively phagocytic macrophages usually appear after 2 or 3 hours. Fibroblast-like cells are seen in the zone of migration close to the explant after 24–36 hours but are usually obscured by the mass of thymocytes. Granular leukocytes, and in some cultures myelocytes, come out in varying numbers in the early hours after planting of the cultures. In addition there are great numbers of dead cells, mostly thymocytes, which apparently resulted from the trauma associated with planting.

Thymocytes. As soon as the culture is warmed in the incubator actively ameboid thymocytes can be seen far out in the clot. Though they are principally small and round, with very little cytoplasm, some are larger cells which correspond in size and appearance to the medium and large lymphocytes of the lymphatic tissue. They are all characterized by relatively large nucleus, large irregular nucleolus or nucleoli, definite nuclear membrane and usually homogeneous cytoplasm, although occasionally small granules or vacuoles are present.

The motion of the thymocytes corresponds in every detail to that of the lymphocytes from control cultures of rabbit lymph node, and to DeBruyn's ('44, '45, '46) descriptions of moving lymphocytes. "Handmirror" shapes with trailing, relatively rigid tail portions are seen in cells moving on the coverglass. "Worm-like" motion is characteristic of cells moving deep in the clot. Here constriction rings are often seen, through which the cell flows while the ring remains stationary. Often a cell, half through such a ring, will squeeze back through and go on in another direction. Such figures strongly suggest that these rings, as DeBruyn contends, are structures in the clot and not part of the cell.

The thymocytes hypertrophy so that as early as 4-6 hours after planting many show filmy, boad processes, very pale and transparent, which flow out from the surface of the cell with a speed difficult to follow even when the motion is only $32 \times$ normal. These films undulate in such a way that they are thrown into folds which are denser and more easily seen, so that at times the folds are the only visible evidence of the films. As the hypertrophying cells begin to show these films, their polarity becomes less pronounced and processes are not confined to the advancing end of the cell. At 12 hours the cells, as seen in the films where their apparent motion is greatly accelerated, glide around with remarkable rapidity and the whipping, darting pseudopodia, which in reality represent the folds of membranes, move with corresponding increase in speed. Irregular inclusions can be seen in the cyto-

plasm of some of the larger thymocytes. Between 12 and 24 hours the number of hypertrophied thymocytes showing ingested particles increases markedly, particularly in the region immediately adjacent to the explant. By 24 hours phagocytic hypertrophied thymocytes are numerous and from this time on they contribute an increasing portion of the macrophages in the cultures.

Not all the thymocytes hypertrophy at the same rate. At 48 hours there are near the explant cells of small thymocyte type with almost no cytoplasm and very small pseudopodia.

Macrophages. The first macrophages appear in the migration zone as early as 2 hours after planting. These large, actively ameboid cells move about ingesting fragments of debris. Their nuclei are large and pale, with vaguely distinguishable nucleoli. The abundant cytoplasm, several times the size of the nucleus, shows numerous irregularly branching processes which often fan out over a broad area at 1 or both ends of the elongated cell body. Between the slim finger-like endings of these processes are stretched filmy undulated membranes similar to those described for the hypertrophying thymocytes. The cells do not at first move around very rapidly in the clot but the processes are very active. The cells exhibit no particular polarity and do not move consistently in any one direction, but the nucleus tends to remain in the trailing portion.

The cytoplasm appears very fluid and the cell membrane very plastic so that large flowing projections can develop and disappear rapidly. As these macrophages accumulate material in their cytoplasm they become more rounded and do not send out long branching processes. They continue to exhibit the undulating membrane type of pseudopodia and actively ingest the cellular debris present in all cultures. At 48 hours most of them are rounded and full of phagocytosed material. Meanwhile the thymocytes have been growing in size and also ingesting much debris so that in some instances it is impossible to say whether a cell was originally a macrophage or thymocyte. This becomes true of many more cells

so that at 4 or 5 days it is no longer possible to make any positive statement about the origin of any of the numerous large globular macrophages present.

The macrophages also exhibit pinocytosis as described by Lewis ('31a, '37) for macrophages and tumor cells in tissue culture. The broad filmy membranes appear to entrap small spherules with highly refractile edges and clear centers, which are drawn into the cell body where they remain visible in the cytoplasm for varying lengths of time. Frequently these spherules were seen to collapse when they came close to the edge of the cell as if the contents were discharged to the exterior.

As stated above, the newly prepared cultures contain numerous dead cells in the zone of migration. In addition smaller particles of debris begin to appear after a few hours and gradually increase in amount. The principal source of this material is the mass of small thymocytes. In addition there is a varying number of erythrocytes. Much of the dead material is localized in the center of the explant where the nutrition is poorest. This material is squeezed out as the tissue contracts so that at 24 hours it is scattered quite generally in the vicinity of the explant and appears in the macrophages of the zone of migration.

Granulocytes. In most cultures a few granular leukocytes can be seen, distinguishable by their specific granules and irregular nuclei. These cells may have come from the blood vessels, remains of which are evident in almost every culture. However, in many cultures there appear in the migration zone cells which appear and behave like thymocytes of the medium and large variety except for the presence in their cytoplasm of granules identical in appearance with those of the granular leukocytes. These cells and the granulocytes exhibit the same motion and the same type of filmy undulating membranes as the macrophages and hypertrophying thymocytes. These early myelocytes, possibly of thymocyte origin, are numerous in some cultures but in others none can be found by observation of living cultures. They occur also in most control sections of explants.

Fibroblast-like cells. These first appear about 36 hours after planting. The mass of migrating cells in the thymus cultures makes their identification and description difficult, but they are beautifully seen in the cultures of subcutaneous connective tissue. They are essentially the same in the thymus as in the subcutaneous tissue cultures and the present description applies to both locations. They appear as free cells moving in very outstretched form. Their thick or thin processes are extremely long, and often branching (fig. 9); some times several rebranchings occur in the fashion of a dendrite. The ends of processes are on the glass, on other cells or free in fibrin. The processes may end bluntly or in bulbs but often end by fading out in the fibrin with no visible border. They may be connected with one another by very fine, almost invisible strands. The edges of these far-flung processes are not usually rigid or tense but show slight or very active "bubbling." The appearance of "bubbling" as seen in the films, results from the relatively rapid development and retraction at numerous places on the cell surface of short, bulbous pseudopods. The extensive processes often run in broad curves and sometimes send branching processes back toward the nucleus. They do not exhibit the filmy, undulating membranes characteristic of the macrophages, nor do they show pinocytosis or phagocytosis. Their nuclei are regularly oval with several clearly defined nucleoli.

The fibroblast-like cells exhibit a type of locomotion not seen in other cell types here described. This locomotion is characterized by a jerking back and forth of the cytoplasm, involving a similar motion of the nucleus. In general the cells move continuously in 1 direction, but at times a cell seems to be moving in 2 directions at once. The flow of cytoplasm may be obvious down 1 process while the nucleus may start down another. Eventually 1 branch becomes the principal one and the nucleus retraces its path and continues with this branch in the main direction of movement. The nucleus often is toward the rear of the cell but long cytoplasmic strands may stretch behind it. Occasionally when the nucleus comes to a

branching it straddles the fork for a time before progressing down one or the other branch.

The "bubbling" edges of the long branching processes are most characteristic. Often bits of such processes are attached only by a slender thread of cytoplasm. They may remain "bubbling" independently for a long time even while the remainder of the cell undergoes mitosis. Occasionally these bits may be torn away completely to become globular particles of debris (elasmotosis).

The migration of free fibroblast-like cells decreases in the older cultures. They tend to settle down into relatively stationary outstretched cells connected to other cells by numerous processes. These cells become very numerous in some cultures, forming an intertwining, many-layered sheath around the whole culture.

In general the appearance of fibroblasts and macrophages conforms to the description of these cells by Carrel and Ebeling ('26).

Consolidation and outgrowth of epithelium in thymus cultures. The epithelium grows in a variety of forms under the varying conditions of life in vitro. It forms tongues, sheets, cords, free spindles, long spindle-shaped chains, broken sheets, and free round cells. These latter are typical free epithelial cells with the exception that on 2 occasions a cell develops from an epithelial mitosis, but moves in a manner somewhat suggestive of a lymphocyte. In both instances the oxygen supply in the culture being photographed was probably quite low.

The first epithelial sheets can be distinguished as early as 24 hours after planting. They frequently lie at the highest plane of focus, close to the cover glass or directly on it. They consist of polygonal cells with exceedingly pale, abundant cytoplasm. The nuclei of the epithelial cells are oval, faintly outlined, and contain several rather prominent nucleoli. The cell outlines are clearly visible. The attachment between the cells is in no sense permanent for the cells, as seen in the films, are continually sliding past one another. Cells occa-

sionally round up, undergo mitosis, and then settle down in the sheet again. Mitoses also occur in outstretched cells. Intercellular bridges are not seen. In older cultures the epithelial cells accumulate vacuoles of faintly pink color and considerable fat. Thymocytes are frequently seen on the sheet or lying in holes in the cytoplasm of the epithelial cells.

These epithelial sheets grow out rapidly. The mitoses are most numerous at the advancing edge of the sheet. Often the advancing edge is composed of cells more loosely bound and showing irregular processes reaching out into the fibrin. These are spindle-shaped cells roughly resembling fibroblasts. In other sheets the edge appears rolled under, thick and highly refractile, composed of contracted cells. These sheets exhibit the properties ascribed to epithelial growth from other organs in the body. The adjacent edges of cells are everywhere in immediate contact. The separation of free cells morphologically identical with fibroblasts at the free edge of the sheet has been described as occurring in most epithelial growth (Levi, '34).

In the main body of the explanted piece, usually at the edge but often more centrally located, dense highly refractile areas begin to appear about 36 hours after explantation. These when sectioned (fig. 13) resemble thymus anlagen in young rabbit embryos. Furthermore, they resemble the masses of epithelial cells which result from the liquefaction and agglomeration of the sheets.

In many cultures, usually after several days *in vitro*, there occurs a growth of cells in some ways similar to but in many ways quite different from the epithelial sheets so far described. The arrangement is that of a sheet but the cells resemble the spindle cells which lie at the periphery of the epithelial sheets. They string out in long chains and remain close together so that the effect is that of a compact sheet of cells. Such sheets do not always grow on the glass surface but may be found deeper in the clot. In places the typical epithelial growth and this peculiar type lie one above the other. These growths do not frequently give rise to free cells, but when they do the

cells are of the epithelial type. In certain places the typical epithelial type of growth merges into spindle cell growth which may become quite expansive, spreading out over an area of several square millimeters. These growths look so much like connective tissue that at first they were considered as such. Pure cultures from such areas, however, grow and behave like the pure cultures of epithelium.

Epithelium in pure culture. Tongues (figs. 1, 2) or unbroken sheets were selected for cutting except on 1 or 2 occasions when the advancing edge of the sheet showed a tendency to form a few free spindle-shaped cells. Such sheets, however, had been under observation long enough to be certain that the spindle cells were epithelial cells from the edge of the sheet and not connective tissue.

In pure cultures from the cut-off tongues, there is first a quiescent period while the cells recover from the trauma of cutting. The new plasma and extract, added to the culture after all but the small tongue of epithelium is cut away, form a new clot over the old one. The new clot is much less dense and apparently more favorable for growth than the old clot in which the bit of tissue lies. There is, therefore, a difference in the growth at the tip of the tongue from that at the cut margin which abuts directly on the new clot. At this cut edge the growth is rapid. The cells undergo numerous mitoses and a sheet of flattened cells spreads out on the cover glass (fig. 6). The cells show a tendency to follow along the cut edge of the fibrin. At the other edge of the cut piece which lies in the denser fibrin (figs. 3, 4) the type of growth is different. Mitoses are also numerous but the sheet breaks up into free cells which move away gradually into the fibrin. In moving they assume various shapes — spindle, round, and branching. Many of the spindle cells remain connected but the regular epithelial arrangement is lost. The spindle shape results from a stretching out of rounded cells. They are under considerable tension and often snap back into round shape when the end of a process loses its contact on the fibrin or on another cell. At other times these processes stretch out into

long, fine strands. A peculiar bubbling of the cytoplasm, apparently not related to the movement of the cell, occurs usually just after the cell rounds up. It is similar to the bubbling that occurs in cells of many types just before mitosis. These free cells in the rounded condition are in every way similar to the cells which result from the breakup of the epithelial sheets to be described below.

Those pure cultures where the cells are growing in thin sheets on the cover glass usually break up in a characteristic manner. As the epithelial cells liquefy the fibrin, connections between cells throughout the sheet appear looser. Many free cells are liberated which may move about, reattach to the sheet, and break free again. These are dense, round, refractile cells with regular, round or oval, centrally located nucleus and single prominent nucleolus. The cytoplasm may be filled with rapidly oscillating fine granules. They move about slowly, retaining for the most part their rounded shape, but frequently with a short, usually branched pseudopodium at the advancing edge of the cell.

Those cells that do not round up stretch out in long chains. At the junctions of the chains, clumps of cells gather as other outstretched cells rapidly contract into rounded cells (fig. 8). These outstretched cells are sharply outlined and refractile, with nuclei like the free cells. They do not show bubbling at their edges in the outstretched condition, in contrast to fibroblasts, but do bubble when they contract down into round cells. The result, after several days, is a liquefied area in the fibrin medium in which are floating numerous free round cells, and clumps of dense, round, concentrically arranged epithelial cells, superficially resembling the Hassall's corpuscles. The centers of these clumps are too dense for detailed study. Occasional epithelial cells remain stretched out flat on the cover glass.

The character of the cells in pure cultures of thymus epithelium is altered considerably after a few days in vitro. Sheets and tongues no longer form and the principal manifestation of the original epithelial origin is the formation of

anastomosing and branching cords of cells, usually about 2 or 3 cells across. The edges of these cords are smooth and unbroken but at the free ends there are spindle cells not unlike those of connective tissue, suggesting contamination by connective tissue. However, they appear consistently in cultures where the possibility of contamination has been excluded, as in the tongue previously described.

In pure culture of thymus epithelium after a week or more *in vitro*, when a certain amount of liquefaction of the fibrin has taken place, many epithelial cells are stretched out directly on the cover glass and show definite filmy membranes. These are relatively permanent structures compared to those of the macrophages but they do undulate and are thrown into folds which move across the membrane in a manner very similar to the films of the macrophages. Pinocytosis by these membranes is also observed and occasionally particulate matter is trapped and forced toward the cell body. In neutral red stained cultures very little dye is taken up by any of the cells. The small round "bubbly" cells take up more than the outstretched ones but no free cells are seen that contain great amounts of dye as do the free macrophages of the connective tissue. In the Janus green cultures small granular and rod-shaped mitochondria are stained in the peripheral clear area of the cytoplasm.

Control cultures of pure gall bladder and intestinal epithelium are essentially similar to those of thymus epithelium, and need not be described. Undulating membranes or pinocytosis were not observed, however.

In photographing cultures, the best results were obtained when the culture on its small cover slip was inverted over a shallow ground depression in a relatively thin slide. The depression was first filled with Tyrode solution to minimize distortion of light by the curved surface of the depression and by condensing moisture in the air chamber. As a result of this procedure the oxygen supply to the tissue gradually diminished during the photographing.

Under such conditions on 2 occasions in different culture series a striking phenomenon was photographed. An epithelial cell, exactly similar in appearance to the others in the cords, rounded up, underwent a mitosis of peculiar violence in the telophase, resulting in 2 daughter cells which differed markedly from each other (fig. 5). One was an ordinary epithelial cell and returned to outstretched form and settled down as part of the cord. The other in both cases was a darker cell, somewhat smaller in diameter (about $9-10\mu$, rather than $12-14\mu$) and moved in a manner entirely foreign to the other free rounded epithelial cells. The nucleus was made out with difficulty in one of them, but was seen somewhat more clearly in the other; it was quite large with respect to the cell, the cytoplasm forming not much more than a rim about it. The nucleolus was large and irregular. The movement bore a striking resemblance to the type of movement seen in lymphocytes. Constriction rings occurred through which the cell squeezed. The cytoplasm appeared to be more fluid than that of the rounded epithelial cells and sprawling, irregular pseudopodia flowed out from the cell in a manner suggestive of similar pseudopodia in cells of the lymphocyte series. The speed of the movement, however, was very much slower than that of lymphocytes, appearing as leisurely at $120\times$ normal speed as the lymphocytes are at $32\times$, or even at $16\times$. Neither of these 2 lymphocyte-like cells that developed from the epithelium was seen to revert again to typical outstretched form in the sheet. The first cell was photographed for approximately 2 hours and the second cell for about 5 hours.

A search of other films of pure thymic epithelium showed 130 mitoses in which both daughter cells could be observed for a time following division. A few of these mitoses were as violent as the unusual ones just mentioned and on several occasions just prior to the complete separation, 1 or both of the daughter cells showed a writhing action which vaguely suggested the type of motion shown by these 2 unusual cells. In none of these instances was a free cell produced which resembled the 2 lymphocyte-like cells described above.

Several unusual mitoses were observed in binucleate cells. The 2 nuclei first merged. The chromosomes then lined up in the shape of a "Y" and when the division took place each branch of the "Y" was split lengthwise so that 3 "V"-shaped groups of chromosomes resulted which later formed 3 nuclei. There appeared in some cases to be 3 daughter cells and in 1 case a single large cell with 3 nuclei as a result of the division. In the last case the cell appeared to divide into 3 cells which fused again into 1 large cell. Amitotic divisions were not observed or photographed in any culture.

Later in the oxygen-poor cultures the cells were seen to die. Outstretched cells rounded up and began to bubble with a violence much greater than that displayed at the mitosis of cells. Then the cells shriveled into irregular shapes with numerous pedicles of dark cytoplasm radiating from a shrunken nucleus. Gradually they again assumed globular form, the nuclei were no longer discernible, and large refractile vacuoles were seen in the remains of the dead cells which floated about showing no further signs of life.

Connective tissue cultures. The growth of connective tissue in cultures of subcutaneous tissue of young and of adult rabbits is essentially similar except that at first the rate of growth is considerably greater in the former and the macrophages more active. The 2 main cell types, macrophages and fibroblast-like cells, are essentially similar to those described above in cultures of thymus.

The fibroblast-like cells usually grow singly and in an interlacing mesh-like tissue near the explant, the cells lying across and in intimate contact with one another. They also grow in broad sheets (fig. 7) at certain places where the cells come in close contact with the glass. These areas at first glance suggest that what has been called epithelium in my thymus cultures may be only a particular form of connective tissue growth. Indeed there are many areas in thymus cultures, especially in older stages, that can not be positively identified as epithelial or connective tissue. Typical epi-

thelium, however, has characteristics never shown by connective tissue (see discussion).

Fixed and stained material

The sections of control pieces of thymus taken from the animals used for culturing show no unusual findings. In the cortex the outstretched cells of the reticulum, with pale nuclei containing dust-like chromatin, are nearly obscured by the dense mass of thymocytes, identical in appearance with the lymphocytes of the lymph nodes. In the medulla the stroma is more prominent, and in intimate relation to the Hassall's corpuscles.

In many of the control pieces of thymus, extramedullary myelopoiesis is found. Heterophile myelocytes are particularly numerous at the periphery of the lobules; the nuclei of many of them are distinctly lymphocytic in character.

Cultures in section and whole mount. The appearance and fate of cells in the zone of migration have been described above in the section on living culture. Observations on fixed and stained materials are included primarily to describe the fate of the dense inner portion of the cultures.

The stroma cells at the periphery of the lobules gradually become more prominent and closer together so that at 12-18 hours they assume an almost cuboid epithelial arrangement in many places. However, these cells also have been multiplying at the surface to form a sheath of several layers of spindle-shaped cells. The first epithelial sheets are seen at 18-36 hours to grow out from these areas of cuboidal epithelium and are continuous with the several layers of spindle cells which sheath the explant. These sheets are broad, 1 cell thick, and composed of polyhedral cells, closely applied to one another at all their thin edges (fig. 12). The edge of the sheet may be smooth and regular or rolled back slightly on itself. Many mitotic figures are seen throughout the sheet. These sheets are usually at the highest focus in the whole mounts but several may be lying one above the other.

The consolidation of epithelium within the explant results at 2 or 3 days in solid areas of dark cells with dense blue-staining cytoplasm, oval or kidney-shaped nuclei and prominent, usually single, nucleoli (fig. 13). These are not sheets but solid islands, several cells thick in each dimension. Such dense accumulations may also form a ring around the outside of the culture so that at 5 or 6 days there may be a ring of epithelium surrounding an area of connective tissue. The epithelium even at this late stage is still permeated by numerous thymocytes apparently moving between the cells. These thymocytes are also seen in the cytoplasm of the epithelial cells, but apparently still alive.

The sheets are not seen in any place to be continuous with the solid condensations of epithelium. Where these latter lie at the periphery of the explant their edges are smooth and contracted. The fibrin is liquefied in these areas, suggesting that the liquefaction prevents growth of sheets.

Many of the cells in the center of the explant, particularly the thymocytes, die very early so that at 12 hours there is considerable dead material here, largely composed of pyknotic nuclei of small thymocytes. As early as 4 hours after planting large phagocytes are seen in these centers which quickly become loaded with the dead material. These cells have nuclei like the stroma cells and like the adventitial cells of the capillaries. Evidence for their formation from either source is not conclusive but their large number, general distribution and relatively dense and abundant cytoplasm suggest an origin from the stroma cells. Some of these phagocytes grow to tremendous size and many come to lie in holes in the epithelial consolidation. Eventually their nuclei shrink and darken.

From areas rich in connective tissue a luxuriant intertwining meshwork of spindles may grow out, completely surrounding the culture. Such connective tissue growths may intermingle with, and be indistinguishable from, sheets of spindle cells which have grown out from the stroma of the thymic lobules. Among the connective tissue cells are out-

stretched phagocytes with large amounts of ingested material. The epithelial cells are not definitely phagocytic although they do accumulate globules of pink material. This latter material is amorphous or finely granular but not hyalin and is stained very much like the red blood cells. However, whole erythrocytes are not seen in these cells and some of the globular inclusions are too large to be remains of red blood cells. They resemble condensed remains of old fibrin clot.

Where epithelial sheets have liquefied the fibrin, many cells adhere closely to the cover glass. Some are evidently connective tissue cells and in places have formed multinucleated giant cells. Others are more like epithelial cells and are closely applied to one another. Around the edges of the liquefaction holes can be seen dense balls of epithelial cells. These when sectioned show both dead cells and mitotic figures (fig. 14), but no changes peculiar to Hassall's corpuscles. The latter are found only in cultures containing portions of the original explant.

Blood vessels are included in all cultures to a varying extent. Sprouts of endothelium grow out much as in granulation tissue. Smooth muscle remains in the vicinity of the vessels for the most part, but after a few days the regular arrangement is lost.

In the fixed and stained pure cultures of thymus epithelium in which the fibrin had liquefied, the main mass of cells is clumped in 1 area and a few cells lie around the periphery of the liquefaction hole. These may be flattened against the cover glass, showing broad, thin, transparent cytoplasmic processes with slightly ruffled peripheries. These resemble the filamentous undulating membranes of living epithelial cells. The cytoplasm directly around the nucleus is dense blue-staining, the nucleus oval or round with very prominent, usually single, nucleolus. Of the outstretched, unliquefied cultures, only those which had been for a week or more in vitro were fixed. In these the cells resemble connective tissue cells, although occasionally they lie in cords and small broken sheets, and have the deeply staining cytoplasm and prominent

nucleoli characteristic of epithelial cells. Giant nuclei and multinucleated cells are frequently seen. There is no evidence of phagocytosis by the epithelial cells, nor are lymphocyte-like forms to be found.

Special stains. Those cultures prepared by the Kopsch method show in the epithelial cells a dense blackening which presumably is the Golgi net, usually close to the nucleus and often concentrated at 1 end in a ball shape (fig. 15). In some cells it is more dispersed through the cytoplasm but is never located very far from the nucleus. In sheets of epithelium this structure usually lies on the side of the nucleus toward the growing edge. The net in the fibroblast-like cells is similar in appearance and offers no criteria by which these 2 cell types can be distinguished.

In Regaud-fixed material stained with iron hematoxylin, thin thread-like, granular and rod-shaped mitochondria are seen in the cytoplasm away from the nucleus. The majority are threads. Although there are great differences from cell to cell, these do not constitute criteria on which to base a distinction between the fibroblast-like cells and the epithelial cells.

DISCUSSION

Two principal problems will be discussed regarding the structure of the thymus. They concern (a) the nature and origin of the stroma and (b) the nature and origin of the small cells lying in its meshes. Connective tissue participation in the structure of the gland, and the origin of the Hassall's bodies will be considered briefly.

All authors agree that the gland has an entodermal origin, principally from the third pharyngeal pouch. Some authors (Prenant, 1894; Bell, '05) hold that this epithelium forms the stroma and gives rise to the thymocytes. The latter are held to be lymphocytes. Beard (1894, '00), on the other hand, derives the stroma from the epithelium but holds that it differentiates very early into lymphoid cells and should be compared directly to the reticulum of the lymph node. Stöhr ('06) agrees with Prenant and Bell as to the origin of the

small cells but considers them to be rounded epithelial elements and not lymphocytes. Among those who favor Stöhr's concept of the epithelial origin of the small cells are Dustin ('20), Schridde ('23), Jaffe ('26), and Gottesman and Jaffe ('26). von Ebner ('02) considers the stroma of the cortex and the lymphocytes to be of connective tissue origin and the medulla of epithelial origin.

Another group (Gulland, 1891, and others) derives the stroma and the small cells entirely from in-growing connective tissue and the epithelium remains for them only in the Hassall's corpuscles. Salkind ('12) speaks of 2 different types of reticulum, one epithelial and the other of connective tissue, and considers the latter to be the source of the lymphocytes.

Hammar ('05, '08) is first to advance the idea of a completely epithelial reticulum with small cells of connective tissue origin in its meshes. The source of these lymphocytes is a massive migration from the surrounding mesenchyme and proliferation within the gland, forcing the epithelium into a loose stroma. Supported by the work of Hammar's student, Jonson ('09), by Söderland and Backman ('09) and later by extensive work by Maximow ('09b, '12a, '12b) the so-called Hammar-Maximow or "lympho-epithelial" theory came into being and found wide acceptance.

Tissue culture in the hands of Wassen ('15), another student of Hammar, and Pappenheimer ('13) reveals that there are cells in the thymus which grow out in a manner resembling epithelium, and that the thymocytes resemble lymphocytes in their mode of motion. More complete investigations by Popoff ('26) and Tschassownikow ('26, '29) have yielded similar results. Both used young rabbit thymus and followed the culture method of Maximow and both introduced lithium carmin in some cultures. These investigators conclude that there is an epithelial reticulum in the thymus, that the thymocytes look and act like true lymphocytes and undergo transformations typical of lymphocytes. The epithelium is held to be non-phagocytic and was not observed to give rise to lympho-

cytes or to phagocytic cells. Both describe the occurrence of 2 types of growth from the cellular reticulum consisting of epithelial sheets in some areas and in others a growth of spindle cells much more characteristic of connective tissue. Tschassownikow considers this suggestive of 2 types of cellular reticulum. Popoff considers it to represent 2 modes of growth of a single epithelial reticulum.

Deanesly ('29) describes a series of cat thymus cultures in connection with an experiment on involution of the thymus after x-ray. She concludes on the basis of morphologic similarity that the spindle cells which form a sheath around her cultures are connective tissue cells. She describes no growth resembling epithelium although she mentions Hassall's bodies forming in "isolated bays projecting from the implant into the plasma." She concludes that the round cells, or thymocytes, originate from the epithelium of the original entodermal anlage. In regeneration these small cells reform from mitoses of the few remaining thymocytes. The rest of the gland, including the Hassall's bodies, is considered to be of connective tissue origin.

Emmart ('36) cultured rat, beef and human embryonic thymus in roller tubes after the method of Gey. She describes 3 principal types of growth: "stroma cells" or connective tissue fibroblasts, "reticular cells" from the reticulum of the gland growing like fibroblasts but at times in sheets of elongate cells, and "epithelioid cells" which form true sheets and balls or "gemmae."

From a study of my cultures in the living condition, in stained sections and whole mounts and particularly from the motion pictures, the following conclusions can be drawn regarding the problems as set forth at the beginning of this discussion:

A. Epithelium exists in the thymus. It grows out in typical form and in pure culture exhibits characteristics shown by other epithelia, namely, those of gall bladder and intestine. It can be distinguished from the fibroblasts and does not give rise to true free macrophages. The origin of this epithelium

directly from the cellular reticulum seems certain. The development of filmy undulating pseudopodia and the observed pinocytosis and possibly phagocytosis by thymus epithelium are characteristics not commonly attributed to other epithelial tissues. The origin of the 2 possibly lymphoid cells from thymus epithelium may be another indication of its peculiar nature.

B. The similarity of appearance and fate of thymocytes and lymphocytes under culture conditions is confirmed, as well as the possibility of formation of myelocytes from typical thymocytes. The modes of locomotion and hypertrophy of the thymocytes correspond to DeBruyn's ('44, '46) descriptions of lymphocytes in moving pictures of lymph node cultures, and also to the behavior of lymphocytes as photographed in my own control cultures of lymph node.

C. Cells capable of rapidly ingesting particulate matter are present throughout the thymus. It is possible that these are epithelial cells. On the other hand, pure thymus epithelium has not given rise to typical macrophages in culture. The large macrophages are present in the earliest sections of cultures (4 hours) and transition forms between them and perivascular cells are suggested but not definite. It is certain that typical macrophages and fibroblasts grow out from the connective tissue septa, but these are not at first large globular cells as are the macrophages in the center of the explant even as early as 7 hours.

D. With regard to the Hassall's bodies the epithelium is seen to form balls of concentrically arranged cells. These never show the characteristic hyalin accumulations or become cystic in their centers, although occasional dead cells are seen. Furthermore such balls can be found in cultures of intestinal epithelium also. Otherwise nothing suggestive of Hallall's bodies is found.

The numerous early investigations on the histogenesis of the thymus are based largely on studies of sectioned embryos of various stages. Repeating such investigations probably will not clarify the problem in view of the thoroughness with

which these lines have been pursued in the past. The method of tissue culture as applied most extensively by Popoff and Tschassownikow yields certain definite conclusions regarding the presence of epithelium and the nature of the free cells. The great value of such cultures lies in the possibility of revealing potencies in cells which they do not ordinarily display clearly *in vitro*. In mixed culture, however, particular cell strains or types are difficult to follow. Transformations (e.g., epithelial cell to macrophage) cannot be followed with certainty in the presence of large numbers of both cell types. Demonstration of a particular property of a cell type (e.g., phagocytosis by epithelial cells) cannot be denied or confirmed in mixed culture where macrophages and epithelial cells might be confused.

The disagreement between Popoff and Tschassownikow regarding the existence of 1 or 2 types of reticulum is a case in point. The conclusion of Popoff that part of the epithelium grows like connective tissue is open to the objection that connective tissue is mixed with it. Conversely the conclusion of Tschassownikow that this growth represents connective tissue is open to the objection that epithelium may at times grow like connective tissue cells. The same applies to Emmart's differentiation between reticular cells, epithelioid cells, and connective tissue cells. The possibility of lymphocytes differentiating from epithelium could never be established for certain in the presence of lymphocytes, connective tissue cells of the septa and undifferentiated perivascular cells.

Deanesly's work as well is open to all these criticisms. She saw no epithelial sheets but does describe bays projecting out from the explant which correspond to the liquefaction holes seen in the epithelium of my cultures and in those of Popoff and of Tschassownikow as well. Hassall's bodies were described by her as lying in these bays. The balls of epithelium in my cultures correspond in location to these corpuscles and even satisfy the criteria she puts forth for Hassall's bodies. It is possible that she has called these balls Hassall's bodies, but in my sections they do not show the characteristic enlarge-

ment and paling of the nucleus and cytoplasm of the central cells, nor accumulations of hyalin. These differences may be due in part to the different animal species used for culturing.

My own experiment in tissue culture is of course open to the general objections regarding the unnatural surroundings forced upon cells in tissue culture, and the consequent limited application to *in vivo* problems of the conclusions drawn from *in vitro* experiments. However, the attainment of a pure strain of cells removes some of the objections raised previously for the mixed cultures of Popoff, Tschassownikow and Emmart. In a pure culture, if we are sure that it is pure, any cell which appears must of necessity bear some cytogenetic relationship to the cell type from which the culture was originally prepared. In addition to this, the application of the moving picture time lapse apparatus makes it possible to follow individual cells over periods of many hours and to watch the actual process of differentiation of cells. These permanent records can be studied many times to observe details that escape an investigator examining living tissue under the microscope directly.

Are there criteria by which we can recognize epithelium in tissue culture? It is well known that connective tissue can assume various forms in tissue culture, among them sheets of closely applied cells which resemble epithelial growth. On the other hand epithelium displays modes of growth not seen in cultures of connective tissue. The most distinctive of these is the "tongue." This mode of growth has several distinguishing characteristics. The edges of the tongue are smooth and everywhere continuous. The cells composing a tongue are in 1 layer, though the tongue may be several cells wide, and are polyhedral in shape with all the edges of the cells adjoining other cells or forming part of the smooth border of the tongue. This conforms in some respects to the characteristic arrangement of some epithelia in the body. Very similar to the tongues are broad sheets with 1 layer of polyhedral cells and smooth continuous borders. These sheets are in a sense

only wide tongues. In the case of the tongues, however, it is possible to photograph for a time the exact cells which are later to be cut away to be cultured as pure epithelium. This was done on 3 occasions. It is possible in the photographs to follow each cell in the field and determine that it is part of the epithelium of the tongue. Thus the possibility of even 1 connective tissue cell creeping in along the edge or between or beneath the cells can be eliminated. The events that occur in cultures cut from tongues duplicate the events in cultures cut from sheets in every detail with 1 exception. The formation of the 2 peculiar, lymphocyte-like cells occurred only in cultures cut from sheets. However, the possibility of these cells representing contamination is eliminated entirely by the fact that they result from mitoses of cells which come from the epithelial cords, and 1 daughter cell in each mitosis subsequently is included in the cords. In the pure cultures prepared from sheets, moreover, the area to be used is carefully examined cell by cell before it is cut away.

Having established that the epithelium is pure we must also be sure that it is pure thymus epithelium. Possible sources of contamination are the mesothelium, the endothelium of the blood vessels and the embryonic extract used. The mesothelium was eliminated by the method of securing the culture tissue as previously described. The endothelium was seen in many cultures to be growing out in typical capillary sprouts but never was seen to form sheets or tongues. The embryonic extract, while not frozen or filtered, was always thoroughly centrifuged and control cultures were made of extract and plasma alone which on only 1 occasion showed growth of a few fibroblast-like cells. Isolated areas of embryonic growth were not seen in the fibrin around the explant. More direct evidence is the definite continuity of the sheets in the sectioned cultures with the cells of the reticulum of the thymus lobules.

Whether the epithelium which grows out in thymus cultures comes from the stroma throughout the lobules, or only from epithelial remnants in the medulla, cannot be decided entirely on the basis of this material. But it has been shown

(Rudberg, '07; Tschassownikow, '29; and others) that when the thymus is depleted of thymocytes, the stroma is revealed as a sheet of epithelioid cells, continuous from cortex to medulla. I have confirmed these observations ('46) in extensive material with a great variety of irradiations from external and internal sources. There was no evidence in my preparations of a distinctly different epithelium in the medulla. It would appear, therefore, that the stroma of both cortex and medulla is largely epithelial in nature and gives rise to broad epithelial sheets and tongues in tissue culture.

The possibility that this thymus epithelium is not altogether similar to other entodermal derivatives is suggested by the observation of the peculiar mitoses, and of the development of undulating membranes on the epithelial cells. No such developments are seen in my few cultures of gall bladder or intestinal epithelium and have not been described for these or other epithelia to my knowledge. The conditions under which these phenomena are observed are somewhat unusual. In each case there is a definite possibility that the oxygen supply was low due to the culture having been immersed in the shallow Tyrode-filled depression for 12 hours or more. The lymphocyte-like cells probably do not represent dying forms, for the death of cells in these cultures was observed later and occurred in a very characteristic fashion. Moreover, these 2 cells continued to move about with no apparent further change as long as observations were continued, in 1 instance 2 and in the other 5 hours after the mitosis. The connective tissue-like growth of thymus epithelium does not stamp it as peculiar, for such growth is described by Champy ('12) for various epithelia in culture. There is no real evidence that this constitutes a true dedifferentiation as Champy thought.

It seems quite certain that thymocytes transform into macrophages. The objection can be raised that single cells are not followed through the entire course of hypertrophy. It has not been possible under the conditions of the experiment to keep a single moving thymocyte in focus for a period of a day or more as would be required to overcome this objection. Con-

sequently only the general process can be observed in the various cells that came under observation. In this connection, however, the great mass of evidence is in favor of the identity of the lymphocyte and thymocyte. This includes, in addition to their morphologic similarity, the formation of myelocytes from thymocytes in animals with spleen grafted into the allantois (Danchakoff, '16), the similar reaction of these cells to x-ray (Rudberg, '07, and others), and the fact that both are cytolysed by sera obtained from rabbits injected with rat thymocytes (Pappenheimer, '17).

If it could be proved that the lymphocyte-like cells which develop from the epithelium are true lymphocytes, it would strongly suggest that the thymocytes originate from the epithelium in the first place. And if lymphocytes can form from cells of entodermal origin the concept of the relative specificity of the germ layers will require further examination in this light.

The film leaves no doubt that the mitoses by which these 2 lymphocyte-like cells arise are differentiating mitoses in some sense. There is in each case a cell formed which, regardless of whether or not it is a lymphocyte, is remarkably different from the other daughter cell. Differentiating mitoses have been described in several situations. The division of the ovum with formation of the polar bodies is an example of this rare phenomenon. Several of the investigators who believe in the epithelial origin of the small thymocytes describe differentiating mitoses by which epithelial cells give rise to 2 small round cells (Prenant, 1894; Dustin, '20). Sabin ('17) describes angioblasts in living chick embryos dividing to give an erythroblast and an angioblast.

Maximow ('09a) considers differentiating mitoses with similar daughter cells to be important in the differentiation of blood cells in the blood-forming tissues. He frequently finds the first specific granules in cells just at the telophase of the mitosis. The mitoses which produce the 2 lymphocyte-like cells in my cultures have a violent and protracted period of telophase.

SUMMARY

In cultures of rabbit thymus, growths of compact tongues or sheets of epithelium appeared. These were grown in pure state by cutting away the rest of the culture, and their activity and fate observed directly, and by time lapse cinephotomicrography. Stained sections and whole mounts were also examined. The results can be summarized as follows:

1. Epithelium probably constitutes most of the cellular reticulum of both cortex and medulla in the thymus.

2. This epithelium in pure culture does not give rise to macrophages. Except for the occurrence of pinocytosis and possibly phagocytosis, and peculiar mitoses (see below), it resembles gall bladder and intestinal epithelium obtained in pure culture by the same method.

3. On 2 occasions a cell, in some respects resembling a lymphocyte, resulted from what appeared to be a differentiating mitosis of an epithelial cell. The implications of this phenomenon, which occurred only under unusual culture conditions, are briefly discussed.

4. Thymocytes move like lymphocytes, hypertrophy into macrophages in the same way as do lymphocytes, and presumably are true lymphocytes.

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PLATE 1

EXPLANATION OF FIGURES

1 Tongue of epithelium from a culture of day-old rabbit thymus on the sixth day in vitro. $\times 470$.

2 Tongue of epithelium from a culture of 3-week rabbit thymus on the fifth day in vitro. Arrow indicates rounded cells in mitosis. $\times 470$.

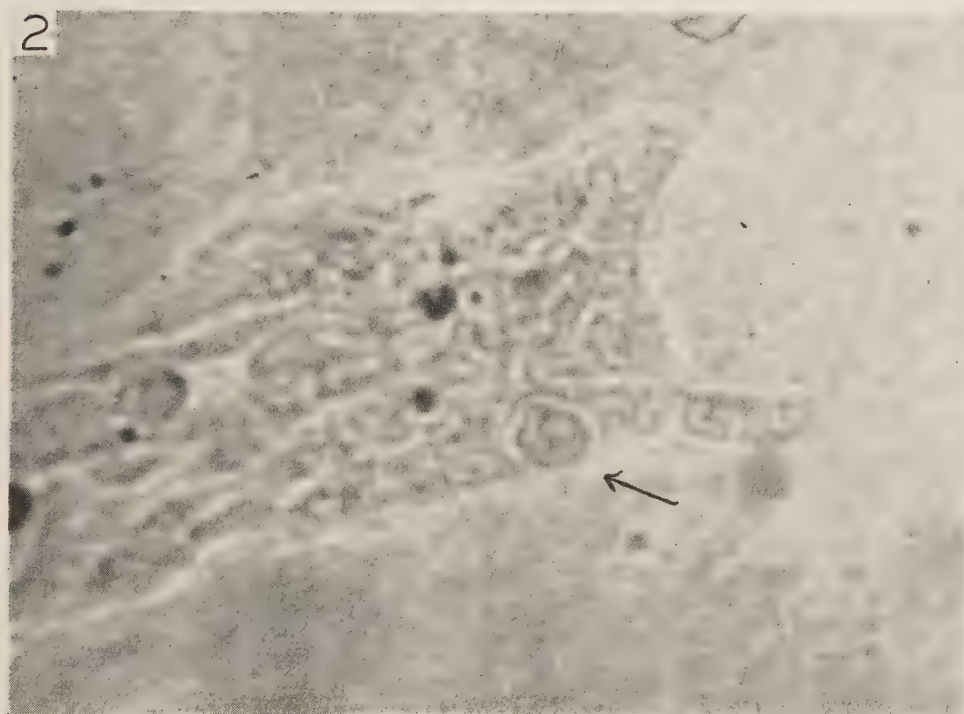


PLATE 2

EXPLANATION OF FIGURES

3 Pure culture of thymus epithelium. Tip of tongue shown in figure 2 on the sixth day in vitro, 4 hours after cutting away the rest of the culture. $\times 470$.

4 Same area as figure 3, 8 hours later. Note elongated, refractile cells detached from the epithelial tongue. $\times 470$.

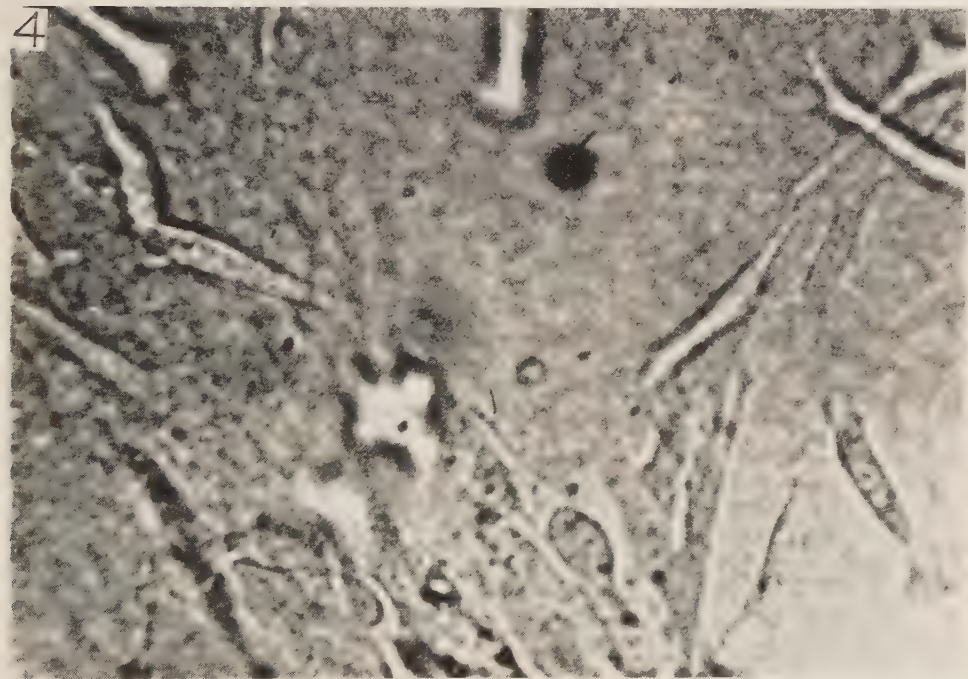
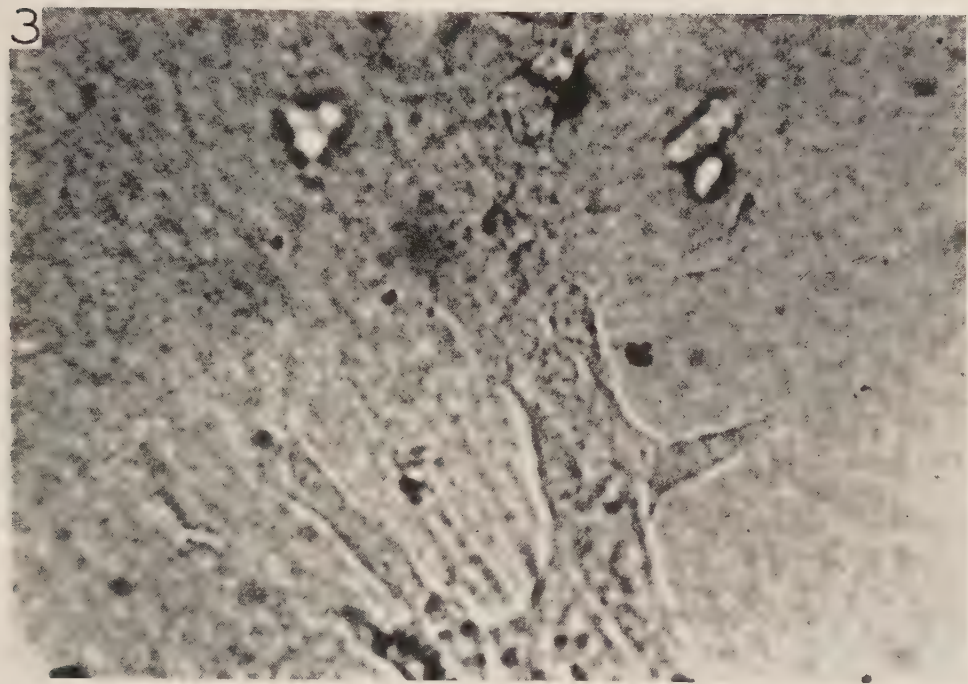


PLATE 3

EXPLANATION OF FIGURES

5 Sequence of enlargements from 16-mm time lapse motion picture of pure culture of thymus epithelium from a 2-day rabbit on the fifth day in vitro. The culture was under increasingly low oxygen tension during photography. The frames show successively: A-C, an epithelial cell (arrow) rounding up; D-F, a violent mitosis; G-L, the unlike daughter cells of this mitosis, the upper one (upper arrow) returning to the epithelial cord and the lower one (lower arrow) moving about in a manner suggestive of the motion of a lymphocyte. Round cell, lower right in K and L, is an epithelial cell in prophase of mitosis. The frames were not chosen at regular intervals. Elapsed time from A to L was 1 hour and 20 minutes. $\times 560$.

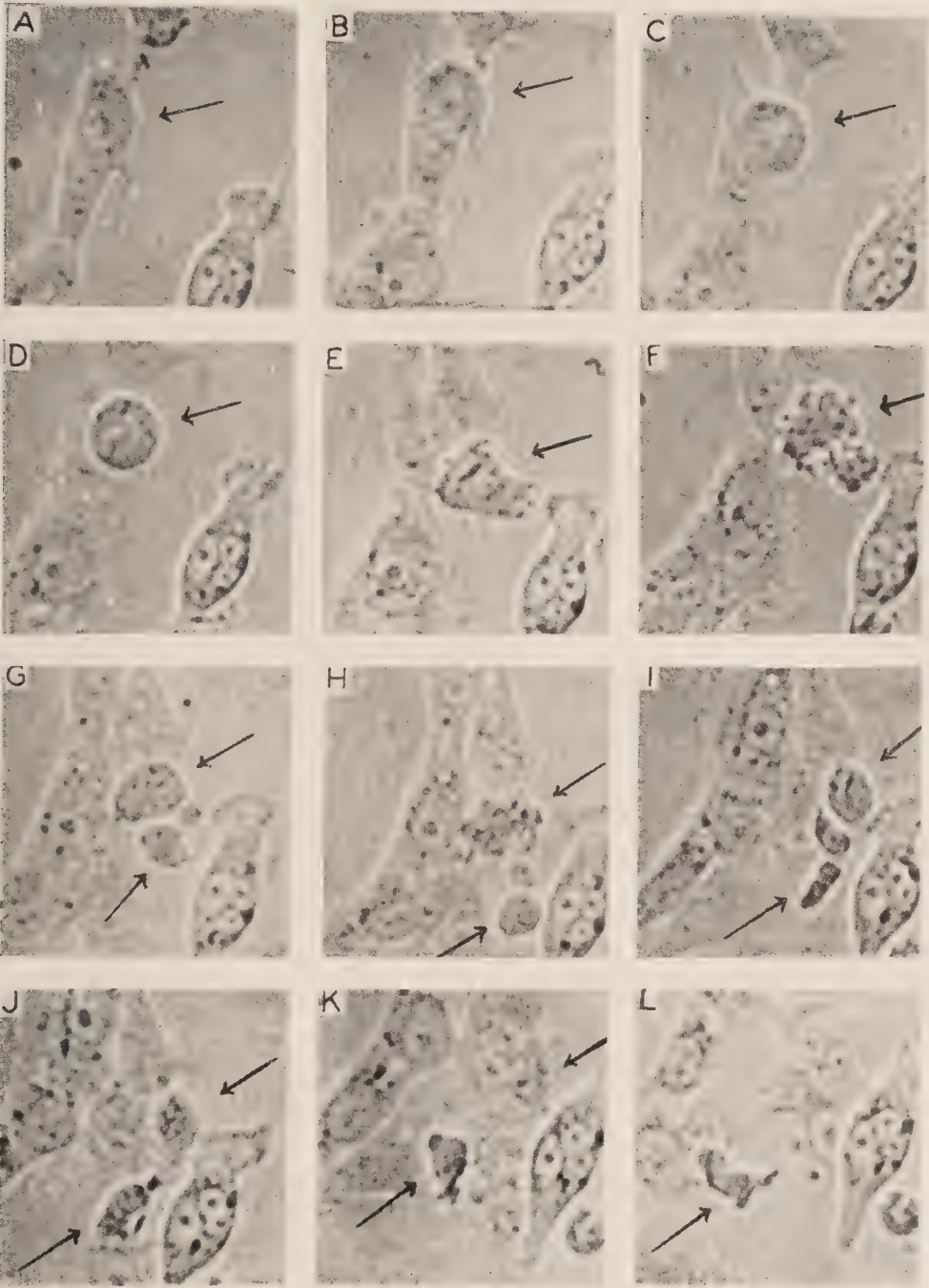


PLATE 4

EXPLANATION OF FIGURES

6 Pure culture of thymus epithelium. Epithelial cells growing out on the cover slip from the cut edge of tongue shown in figure 1. $\times 240$.

7 Fibroblasts from culture of adult rabbit subcutaneous connective tissue on the fourth day in vitro. Cells are growing in a broad sheet. $\times 480$.

8 Liquefied pure culture of thymus epithelium from a young rabbit, sixth day in vitro. Note refractile, rounded cells. $\times 440$.

9 Fibroblasts from culture of 1-day rabbit subcutaneous connective tissue, 24 hours in vitro. $\times 480$.

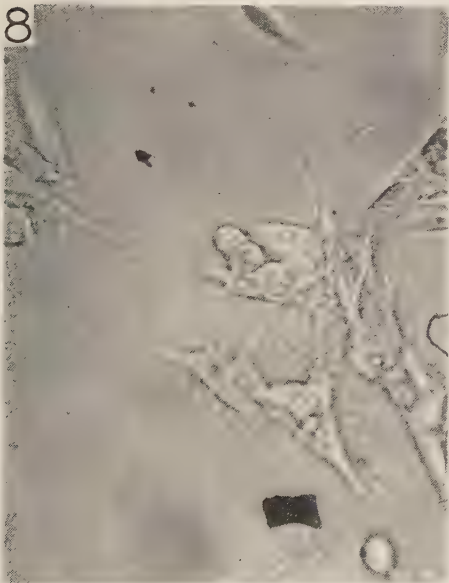
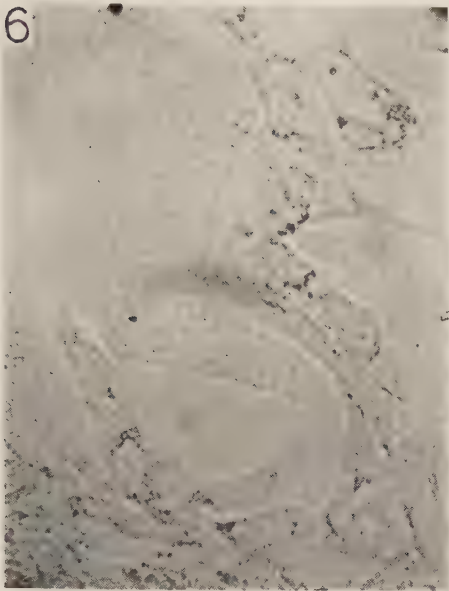


PLATE 5

EXPLANATION OF FIGURES

10 Pure thymus epithelium from a 2-day rabbit on the fifth day in vitro. Rounded free cells are in mitosis, or about to enter mitosis. Larger field from stage F of the sequence illustrated in figure 5. $\times 470$.

11 Pure culture of gall bladder epithelium from 20-day rabbit embryo, fourth day in vitro. Irregular cell bottom middle, and prominent round cell middle right, are epithelial cells in mitosis. Compare with figure 10. $\times 470$.

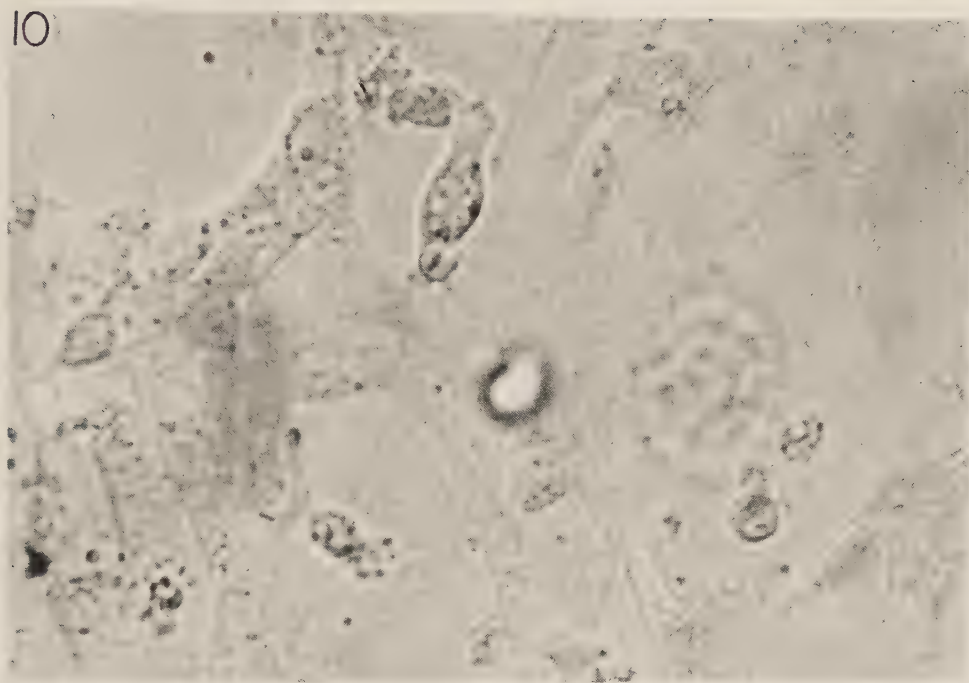


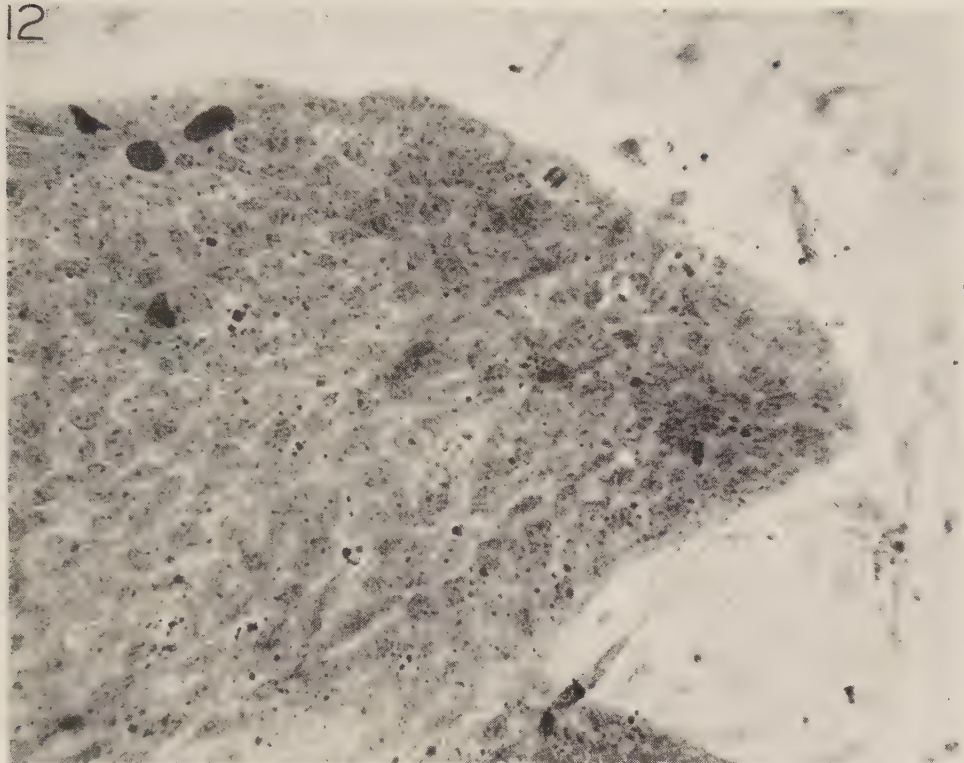
PLATE 6

EXPLANATION OF FIGURES

12 Fixed and stained section of a culture of thymus from a 3-week rabbit, sixth day in vitro. Note broad sheet of epithelium and meshwork of fibroblasts. Hematoxylin-eosin-azure II. $\times 375$.

13 Fixed and stained section of a culture of thymus from a 17-day rabbit embryo, fourth day in vitro. Note consolidations of epithelium within the explant. This resembles a thymus anlage in 14-day rabbit embryo. Hematoxylin-eosin-azure II. $\times 250$.

12



13

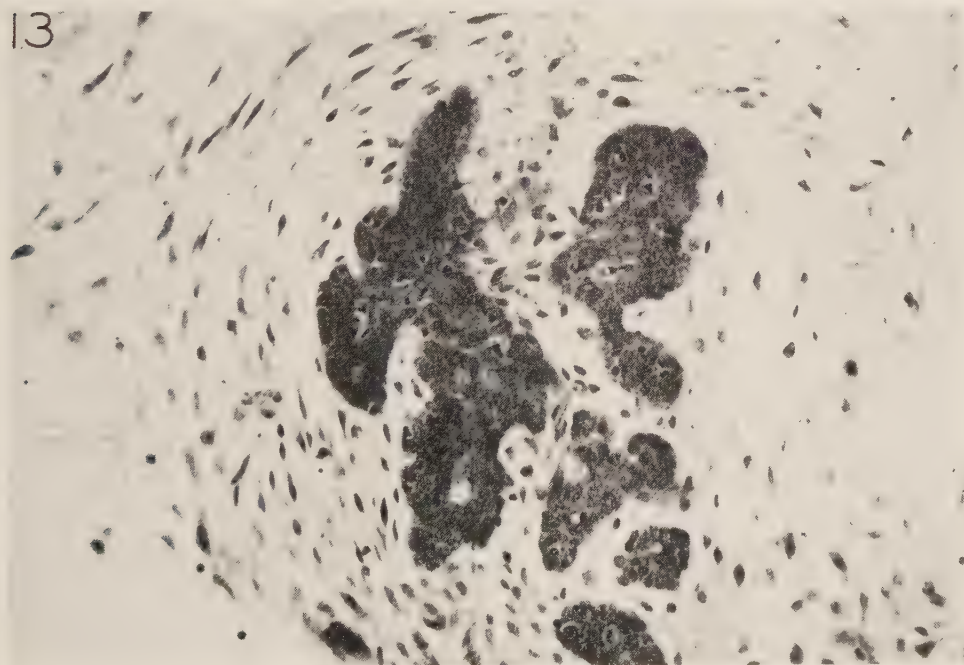


PLATE 7

EXPLANATION OF FIGURES

14 Two fixed and stained sections of a ball of epithelial cells from a culture of 3-week rabbit thymus, twelfth day in vitro. There are dead cells (14a) but no evidence of changes peculiar to Hassall's body formation. Note mitosis at right in 14b. Hematoxylin-eosin-azure II. $\times 750$.

15 Whole mount of a culture of thymus from a 3-week rabbit, eighth day in vitro. Fixed by a modified Kopsch method to demonstrate the Golgi apparatus. $\times 750$.

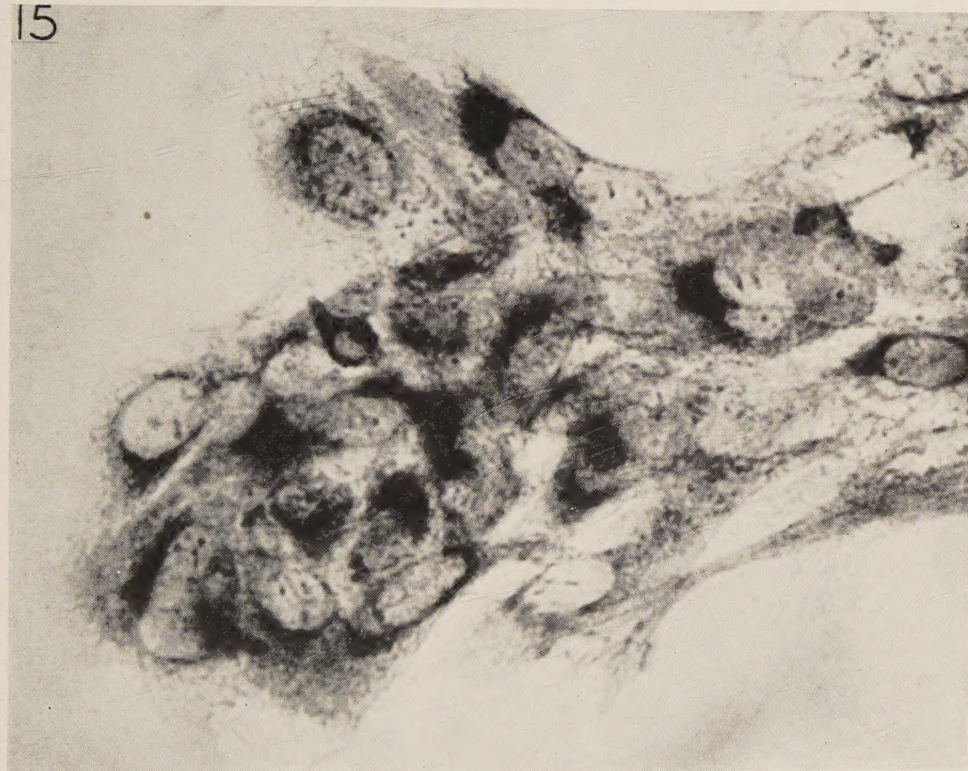
14A



14B



15



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